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13. ABSTRACT ***(Maximum 200 Words)***
In this report we present data in support of Aim 3 of our project. We demonstrate that ectopic expression of wild type or phosphorylation site mutants of 4E-BP1 in breast cancer cells increases apoptosis, reduces the ability of cells to form colonies *in vitro*, and markedly inhibits xenograft tumor growth *in vivo* in a manner dependent on the potency of 4E-BP1 to inhibit cap-dependent translation. During *in vitro* and in *vivo* progression, transfected breast cancer cells tend to lose expression of exogenous 4E-BP1. Silence of ectopic 4E-BP1 is associated with restoration of cell malignant phenotype and chemoresistance. We conclude that intensification of 4E-BP1 phosphorylation and other event leading to inactivation of 4E-BP1 are under strong selective pressure and contribute to tumor progression by providing cell survival advantages. Together, these findings suggest that the sustained activation of the cap-dependent translation apparatus is an important mechanism by which cancer cells evade apoptosis and acquire chemoresistance. Targeted disruption of aberrant cap-dependent translation provides the opportunity to utilize this cancer cell-selective death pathway for anticancer therapy which spares normal cells.

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Table of Contents

Introduction

The functional activity of the translational complex eIF4F is invariably elevated in a broad spectrum of naturally occurring tumors, including breast carcinoma (Raught et al, 2000). Culture- and tumor xenograft-based experiments demonstrate that aberrant activation of eIF4F stimulates cell cycle transit (Raught et al 1999b), rescues cells from apoptosis (Polunovsky et al., 1996, 2000, Tan et al., 2000) and synergizes with pre-neoplastic alterations, such as deregulated c-Myc, in promoting malignant transformation (Raught et al, 2000). Consistent with this, the eIF4F antagonist, translational repressor 4E-BP1, displays attributes of a tumor suppressor. It is functionally inactivated in aggressive breast carcinomas by hyperphosphorylation. These findings support the idea that an aberrantly activated cap-dependent translation apparatus may be a critical determinant of breast carcinogenesis, and that the 4E-BP family of translational repressors may serve as tumor suppressors that are required for breast cancer surveillance.

The objective of this awarded project is to experimentally test the idea that targeted disruption of the anti-apoptotic function of eIF4E can sensitize breast carcinoma cells to therapeutic doses of a non-genotoxic cytostatic agent such as lovastatin and/or to low concentrations of genotoxic agents. We also propose to develop treatment strategies which will include disruption of the anti-apoptotic function of eIF4E in a combination with treatments with non-toxic doses of lovastatin and conventional anti-neoplastic agents. The results of *in vivo* experiments give us a strong rationale for applying a similar gain-and loss-of-function strategy for testing 4E-BPs as candidate suppressors of breast carcinogenesis in the physiological context of a whole organism. Here we present data in support of Aim 3 (Task 3) of our project: Utilize a nude mouse xenotransplantation preclinical model to examine proof of principle: reduction in eIF4E activity chemosensitizes human breast cancer cells.

Body

Aim 3. Utilize a nude mouse xenotransplantation preclinical model to examine proof of principle: reduction in eIF4E activity chemosensitizes human breast cancer cells.

Expermental Data in Support of Aim 3

Translational repressor 4E-BP1 inhibits xenograft tumor growth in a manner dependent on its phosphorylation status.

Our data reported in support of Aim 1 suggest that cells from breast carcinoma lines function in a translationally active state including increased expression of translation factors eIF4GI and eIF4E and inactivation of the translational repressor 4E-BP1 by its extensive phosphorylation (Annual Report 2000). We also provided evidence that targeted suppression of cap-dependent translation due to enforced overexpression of the translational repressor 4E-BP1 activates apoptosis in breast cancer cells and decreases their potency to form transformed foci *in vitro*. Mutating its phosphorylation sites markedly increases the pro-apoptotic potency of 4E-BP1 (Annual Report 2001). These findings suggest that breast cancer cells require sustained activation of the cap-dependent translational apparatus to evade apoptosis and that increased phosphorylation of 4E-BP1 is an important component of the translationally mediated survival signaling.

Since the mechanism by which the translation apparatus operates on the cellular apoptotic machinery remains uncertain, we the also traced the downstream survival pathway pathway that leads to interdiction of the apoptotic program. Experiments achieving gain and loss of function demonstrate that eIF4E-mediated rescue from Myc-induced apoptosis is governed by pretranslational and translational activation of *bcl*-x, as well as by additional intermediates acting directly on - or upstream of - the mitochondria. Results of these studies are published in *Mol Cell Biol* (22: 2853-2861, 2002) and accepted for publication in *J Biol Chem*.

Our next intention was to test the role of 4E-BP1-mediated translational repression in tumorigenesis.

Figure 1

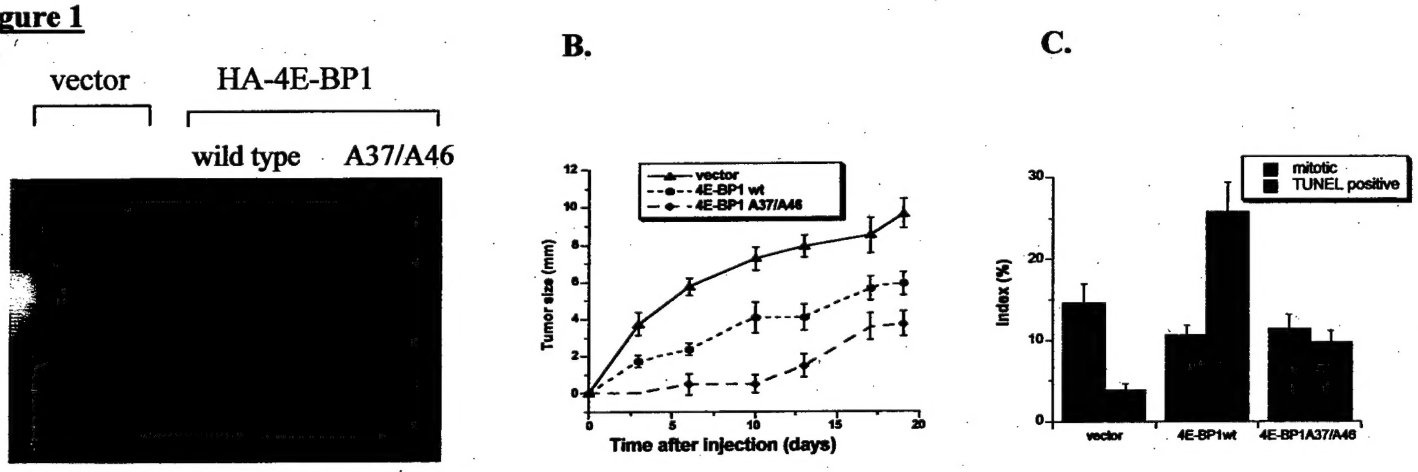

Figure 1. 4E-BP1 inhibits xenograft tumor growth in a manner dependent on its phosphorylation status.
(A) Representative mice injected with transduced MDA-MB-468 breast carcinoma cells photographed at 21 day after injection **(B)** Quantification of tumor size over time.

To detect whether ectopic expression of 4E-BP1 was sufficient to inhibit tumor growth, breast carcinoma MDA-MB-468 cells were transduced with a retroviral construct encoding GFP and wild type or A37/A46 mutant of HA-4E-BP1 and cell populations containing 100% GFP positive were sorted, as described (see Annual Report 2001) and injected into mammary fat pads of immunodeficient mice. Cells transduced with wt 4E-BP1 formed tumors which grew with at a markedly slower rate than tumors formed by vector transduced cells (Figure 1 A and B). Both type of tumors were first apparent at day 3 but at day 20 wt 4E-BP1 overexpressing tumors were approximately half the size of those formed my mock-transduced cells. In stark contrast, MDA-MB-468 cells expressing double phosphorylation site mutant of 4E-BP1 formed tumors that were first apparent after a twelve-day latency. These tumors subsequently grew at rates similar to those formed by wt 4E-BP1 expressing cells. All tumors were excised after euthanasia at day 20, and formalin-fixed paraffin-embedded sections were stained with hematoxylin-eosin (HE) to examine tumor morphology. Tumors formed by cells expressing ectopic 4E-BP1 have fewer blood vessels than control tumors and displayed increased cellular and nuclear polymorphism (not shown).

The mitotic index was slightly decreased in tumors formed by cells transfected by either wild type or mutant 4E-BP1 (Figure 1C). To determine impact of apoptosis in tumor formation, a TUNEL assay was performed. The tumors formed by mock-transduced cells exhibited a low frequency of background apoptosis. In contrast with the results of the *in vitro* assay (Annual Report 2001), expression of wild type HA-4E-BP1 increased the apoptotic index in tumor cells by nearly 7-fold (Figure 1C). Unexpectedly, only two-fold increase of cells death was observed in tumors formed by A37/A46 4E-BP1 transduced cells. We considered the possibility that most A37/A46 4E-BP1 cells have already undergone apoptosis during the latent period of tumor growth and that the tumors arose from those cells that have lost or decreased expression of A37/A46 4E-BP1. Indeed, immunohistochemical assays of tumor sections revealed a marked reduction in intensity of HA staining in tumors formed by mutant HA-4E-BP1 cells compared with the wt 4E-BP1/MDA-MB-468 formed tumors (not shown). Of note, in tumor regions where intensive cell death was observed, cells invariably expressed increased levels HA-4E-BP1. Together, these observations suggest that increased expression of wild type or hypophosphorylated mutant 4E-BP1 decreases tumorigenicity of MDA-MB-468 cells by activating apoptosis. They also show that tumor cells tend to lose the expression of the 'super-repressor' form of 4E-BP1. These findings prompted us to examine a relationship between expression levels of exogenous 4E-BP1 and cell viability in long-term cultivated breast carcinoma cells.

Selection for loss of ectopic 4E-BP1 expression is associated with gain of resistance to apoptosis

To directly test for the loss of ectopic 4E-BP1, we monitored steady state levels of introduced HA-4E-BP1 in non-transformed human mammary epithelial cells (HMECs) and breast carcinoma cells during sustained cultivation *in vitro*. (Figure 2).

Figure 2

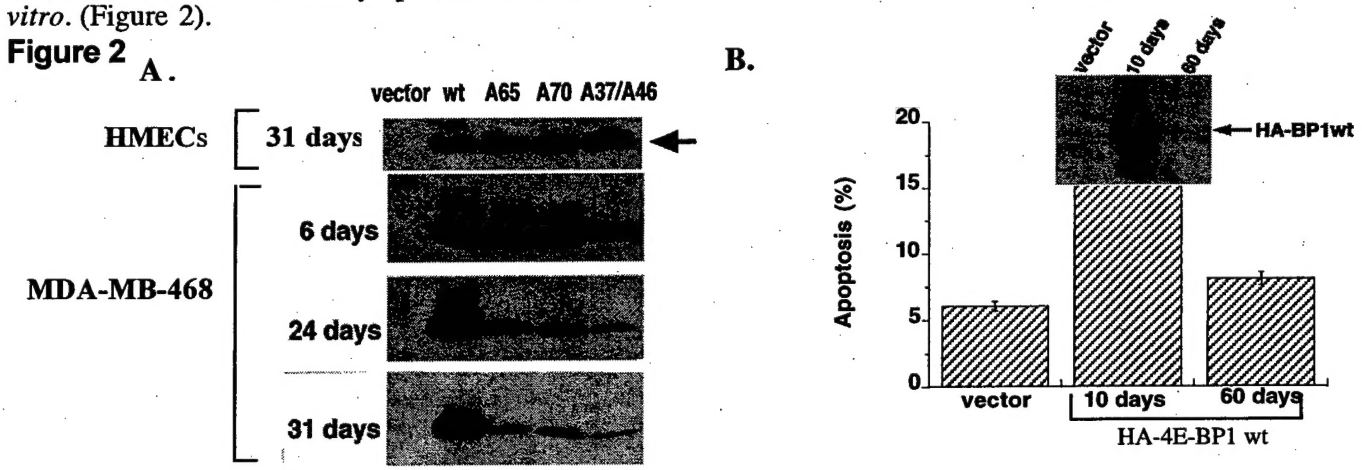

Figure 2. Loss of ectopic 4E-BP1 expression in breast cancer cells correlates with restoration of resistance to apoptosis. **(A)** Expression levels of introduced wild type and hypophosphorylated forms of HA-4E-BP1 in HMECs and MDA-MB-468 cells over time. **(B)** Levels of HA-4E-BP1 and apoptosis in MDA-MB-231 cells transfected with wild type 4E-BP1.

Non-transformed I-HMECs do not undergo apoptosis when they overexpress either wild type or mutant 4E-BP1 (Annual Report 2001). In these cells, expression levels of all forms of exogenous 4E-BP1 were unchanged during 31 days of cultivation (Figure 2A). In contrast, introduction of hypophosphorylated forms of 4E-BP1 into MDA-MB-468 cells results in cell death both *in vitro* and *in vivo* (Annual Report 2001, present communication). When MDA-MB-468 cells ectopically expressing 4E-BP1 were cultivated under regular growth conditions, expression of mutant 4E-BP1s rapidly declined with only traces of A37/A46 4E-BP1 at day 31. Conversely, expression of wild type 4E-BP1, which is non-apoptotic in these cells *in vitro*, remained constant. These findings indicate a possible casual link between the pro-apoptotic activities of the mutant forms of 4E-BP1 and the loss of their expression in cancer cells. Alternatively, different fates of wild type and mutant 4E-BP1s in cultured MDA-MB-468 cells can be attributed to differences between these forms that are unrelated to their pro-apoptotic function. To test this, we traced expression of HA-4E-BP1 in wild type 4E-BP1-transfected MDA-MB-231 cells where increased apoptosis has been documented (Annual Report 2001). After 60 days of cultivation, MDA-MB-231 cells almost lacked ectopic wild type 4E-BP1 (Figure 2B). This loss of ectopic 4E-BP1 was accompanied with restoration of cell resistance to apoptosis indicating the interdependence between the pro-apoptotic function of 4E-BP1 and its loss or silence. Together with results of our experiments with nude mice, these findings are consistent with selection for resistance to apoptosis during *in vivo* and *in vitro* expansion of breast cancer cells by the loss of expression of the pro-apoptotic forms of 4E-BP1.

Discussion

1. We have documented earlier that both the apoptotic and translational machinery are activated in all tested breast carcinomas when compared to non-transformed breast epithelial cells (DOD Annual Report 2000). We have also demonstrated that targeted disruption of the cap-dependent translational complex by ectopic overexpression of the translational repressor 4E-BP1 stimulates apoptosis and abrogates chemoresistance in breast carcinoma cells harboring diverse oncogenic alterations (DOD Annual Report 2000). Here we provide evidence that enforced overexpression of 4E-BP1 in breast carcinoma cells decreases their tumorigenicity in nude mice xenograft by activating apoptosis. In accord with our expectations, we also found that preventing 4E-BP1 phosphorylation by mutating its phosphorylation sites increases the anti-tumor potential of 4E-BP1. These results establish proof of principle: aberrantly activated cap-dependent translation is not only a hallmark of malignant transformation and breast carcinogenesis but also an essential factor required for cancer cell viability and tumor formation. They also support the interpretation that inactivation of 4E-BP1-to-eIF4E binding by 4E-BP1 phosphorylation is required for tumor progression.

2. According our data (DOD Annual Report 2000), increased expression of wild type 4E-BP1 in MDA-MB-468 breast carcinoma cells neither activates apoptosis nor affects cell proliferation *in vitro*. Unexpectedly, we found that overexpressed wild type 4E-BP1 increased tumor cell apoptosis and markedly decreased tumorigenicity of MDA-MB-468 cells in nude mice. This discrepancy between *in vitro* and *in vivo* data may merely be a consequence of a strong pro-apoptotic pressure on cancer cells in tumors where survival factor limitations and other suboptimal or noxious conditions in cell microenvironment contribute to apoptotic stress.

3. It is conceivable that a strong pro-apoptotic pressure from milieu should result in selection of cells that escape apoptosis. Indeed, we found that breast cancer cells tend to lose expression of the pro-apoptotic forms of 4E-BP1 both *in vitro* and *in vivo*. This loss parallels restoration of cell resistance to apoptosis and tumor regrowth. Mutating phosphorylation sites contributes to loss of 4E-BP1 in a rank-order that matches the pro-apoptotic potencies of mutant forms of 4E-BP1. We conclude that intensification of 4E-BP1 phosphorylation and other event leading to inactivation of 4E-BP1 are under strong selective pressure and contribute to tumor progression by providing cell survival advantages.

4. From a therapeutic vantage point, the pro-apoptotic activity of 4E-BP1 would possess several attractive characteristics. First, while ectopic 4E-BP1 increases apoptosis in breast cancer cells, even significant overexpression of wild type or hypophosphorylated forms of 4E-BP1 is nontoxic for non-transformed breast epithelial cells. This provides the opportunity to utilize this cancer cell-selective death pathway for anticancer therapy and to spare normal cells. Second, overexpressed 4E-BP1 cooperates with non-toxic, therapeutically achievable doses of cytotoxic drugs. Third, pharmacological normalization of the aberrantly upregulated apoptotic machinery can be achieved by disrupting association of eIF4E with capped mRNA by small molecules that can interfere with the cap-binding domain of eIF4E. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).

Key Research Accomplishments

• Based on our previous findings (Annual Reports 2000, 2001), we argue that genetic alterations leading to activation of the cap-dependent translational apparatus confer mammary epithelial cells some aspects of malignant transformation. We also postulate that transfer of genes encoding translational repressor 4E-BP1 or its phosphorylation site mutant forms chemosensitize breast cancer cells to safe doses of some conventional anti-cancer drugs, including doxorubicin and the topoisomerase I inhibitors. The results of *in vivo* experiments give us a strong rationale for applying a similar gain-and loss-of-function strategy for testing 4E-BPs as candidate suppressors of breast carcinogenesis in the physiological context of a whole organism.

• Our results suggest that transfer of genes encoding translational repressor 4E-BP1 or its phosphorylation site mutants decreases tumorigenicity of breast carcinoma cells in mouse xenograft model of breast cancer by activating apoptosis. They also show that tumor cells tend to lose the expression of the 'super-repressor' form of 4E-BP1. These findings prompted us to examine a relationship between expression levels of exogenous 4E-BP1 and cell viability in long-term cultivated breast carcinoma cells.

• Our data show that expression of pro-apoptotic forms of 4E-BP1 rapidly declines in cultured breast carcinoma cells. This loss of ectopic 4E-BP1 was accompanied with restoration of cell resistance to apoptosis indicating the interdependence between the pro-apoptotic function of 4E-BP1 and its loss or silence.

• Importantly from a therapeutic point of view, while ectopic 4E-BP1 increases apoptosis in breast cancer cells, even significant overexpression of wild type or hypophosphorylated forms of 4E-BP1 is nontoxic for non-transformed breast epithelial cells. This provides the opportunity to utilize this cancer cell-selective death pathway for anticancer therapy and to spare normal cells. Based on these data, we plan to employ novel pharmacological approaches including disruption of eIF4E-to-cap association by phosphoramidated analogues of the 5' methylguanosine cap of mRNA. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).

Reportable Outcomes

• Manuscripts, Abstracts, Presentations.

Manuscript:

1. Li S, Sonenberg N, Gingras A-C, Peterson M, Avdulov S, **Polunovsky VA**, Bitterman PB. Translational control of cell fate: availability of phosphorylation sites on translational repressor 4E-BP1 governs its pro-apoptotic potency. *Mol Cell Biol,* 2002, 22: 2853-2861
2. Li S, Takasu T, Perlman D, Peterson M, Burrichter D, Avdulov S, Bitterman P, **Polunovsky V.** Translation factor eIF4E rescues cells from Myc-dependent apoptosis by inhibiting cytochrome c release. *J Cell Biol,* accepted.

Abstracts:

1. Avdulov S, Peterson M, Li S, Sonenberg N, Bitterman n, and **Polunovsky V**. Translational control of malignancy and acquired chemoresistance: antiapoptotic function of the translational factor eIF4E in breast cancer cells. AACR Annual Meeting,, April 6-10, San-Francisco, CA, 2002
2. Svetlana A. Avdulov, Shunan Li, Mark Peterson, Nahun Sonenberg*, Peter B. Bitterman, and Vitaly A. Polunovsky. Translational control of malignancy: Antiapoptotic function of the translational complex eIF4F. Int J Mol Med, v.10, suppl.1, S79, 2002
3. S. A. Avdulov, S. Li, D. Burrichter, M. Peterson, N. Sonenberg , P. B. Bitterman, and V. A. **Polunovsky** .Translational control of malignancy and chemoresistance in breast cancer cells: antiapoptotic function of the translational factor eIF4E. The 6th International Symposium "Cancer Detection and Prevention" February 9-12, Pasteur Institute, Paris, France, 2002
4. Avdulov S, Peterson M, Li S, Sonenberg N, Bitterman n, and Polunovsky V. Car-dependent translational control of tumorigenicity and chemoresistance in breast cancer cells. The DOD Cancer Research Program Meeting "Era of Hope", September 25-28, Orlando, Florida, 2002
5. Svetlana A. Avdulov, Shunan Li, Mark Peterson, David Burrichter, Nahum Sonenberg, Peter B. Bitterman, and Vitaly A. Polunovsky. Cap-dependent translational control of malignant phenotype in mammary epithelial cells. The Meeting "Translational Control" September 10-15, Cold Spring Harbor, NY, 2002

Presentations:

1. **Polunovsky VA,.** Avdulov S, Shunan Li, Peterson M, Gingras A-C, Sonenberg N, and Bitterman P. Translational control of malignancy and chemoresistance in breast cancer cells: antiapoptotic function of the translational factor eIF4E. 6th International Symposium "Cancer Detection and Prevention" February 9-12, Pasteur Institute, Paris, France, 2002
2. **V. A. Polunovsky,** S. A. Avdulov, S. Li, D. Burrichter, M. Peterson, N. Sonenberg , P. B. Bitterman, Translational control of malignancy: Antiapoptotic function of the translational complex eIF4F. The 7th World Congress on Advances in Oncology, October 10-12, Hersonissos, Crete, Greece, 2002
3. Avdulov SV, Li S, Peterson M, Gingras A-C, Sonenberg N, Bitterman P, **Polunovsky VA.** Translational control of acquired chemoresistance in breast cancer cells: anti-apoptotic function of the activated cap-dependent translational apparatus. International Meeting in Sardinia: "New Targets in Molecular Carcinogenesis." September 23-26, 2001

• Patents

None

• Degrees obtained that are supported by this research

None

• Informatics such as databases and animal models, etc

None

• Funding applied for based on work supported by this award

Active:

National Institutes of Health

Translational Apparatus as a Target for Cancer Drug Discovery (**V. Polunovsky** PI)

RFA CA-00-002

04/01/2001-03/31/2005 $350,000 Direct cost/year, 40% effort

Pending:

1. Department of Defense

Cap-dependent Translational Control of Human Breast Carcinogenesis and Tumor Cell Chemoresistance
Proposal Category: Idea
09/01/03 – 08/31/06 $100,000 Direct cost/year, 35% effort,

3. Susan G. Komen Foundation

Disruption of breast cancer chemoresistance by the translational antagonist 4E-BP1:A bitransgenic mouse model.
05/01/03-04/30/06 $100.000 direct cost/year

• **Employment or research opportunities applied for/or received....**

Full Professor, University of Minnesota, Department of Medicine

Conclusions

In this report we present experimental data in support of Aim 3 of our project: Utilize a nude mouse xenotransplantation preclinical model to examine proof of principle: reduction in eIF4E activity chemosensitizes human breast cancer cells. Our results show that enforced overexpression of the translational repressor 4E-BP1 in breast carcinoma cells decreases their tumorigenicity in nude mice xenograft by activating apoptosis. In accord with our expectations, we also found that preventing 4E-BP1 phosphorylation by mutating its phosphorylation sites increases the anti-tumor potential of 4E-BP1. These results establish proof of principle: aberrantly activated cap-dependent translation is not only a hallmark of malignant transformation and breast carcinogenesis but also an essential factor required for cancer cell viability and tumor formation. They also support the interpretation that inactivation of 4E-BP1-to-eIF4E binding by 4E-BP1 phosphorylation is required for tumor progression. In line with these observations, we found that breast cancer cells tend to lose expression of the pro-apoptotic forms of 4E-BP1 both *in vitro* and *in vivo*. This loss parallels restoration of cell resistance to apoptosis and tumor regrowth. Mutating phosphorylation sites contributes to loss of 4E-BP1 in a rank-order that matches the pro-apoptotic potencies of mutant forms of 4E-BP1. We conclude that intensification of 4E-BP1 phosphorylation and other event leading to inactivation of 4E-BP1 are under strong selective pressure and contribute to tumor progression by providing cell survival advantages. Since even significant overexpression of wild type or hypophosphorylated forms of 4E-BP1 is nontoxic for non-transformed breast epithelial cells, the pro-apoptotic activity of 4E-BP1 is attractive from a therapeutic vantage point. Targeted disruption of aberrant cap-dependent translation provides the opportunity to utilize this cancer cell-selective death pathway for anticancer therapy which spares normal cells. Pharmacological normalization of the aberrantly upregulated apoptotic machinery can be achieved by disrupting association of eIF4E with capped mRNA by small molecules that can interfere with the cap-binding domain of eIF4E. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).

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Translational Control of Cell Fate: Availability of Phosphorylation Sites on Translational Repressor 4E-BP1 Governs Its Proapoptotic Potency

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Translational control has been recently added to well-recognized genomic, transcriptional, and posttranslational mechanisms regulating apoptosis. We previously found that overexpressed eukaryotic initiation factor 4E (eIF4E) rescues cells from apoptosis, while ectopic expression of wild-type eIF4E-binding protein 1 (4E-BP1), the most abundant member of the 4E-BP family of eIF4E repressor proteins, activates apoptosis—but only in transformed cells. To test the possibility that nontransformed cells require less cap-dependent translation to suppress apoptosis than do their transformed counterparts, we intensified the level of translational repression in nontransformed fibroblasts. Here, we show that inhibition of 4E-BP1 phosphorylation by rapamycin triggers apoptosis in cells ectopically expressing wild-type 4E-BP1 and that expression of 4E-BP1 phosphorylation site mutants potently activates apoptosis in a phosphorylation site-specific manner. In general, proapoptotic potency paralleled repression of cap-dependent translation. However, this relationship was not a simple monotone. As repression of cap-dependent translation intensified, apoptosis increased to a maximum value. Further repression resulted in less apoptosis—a state associated with activation of translation through internal ribosomal entry sites. These findings show: that phosphorylation events govern the proapoptotic potency of 4E-BP1, that 4E-BP1 is proapoptotic in normal as well as transformed fibroblasts, and that malignant transformation is associated with a higher requirement for cap-dependent translation to inhibit apoptosis. Our results suggest that 4E-BP1-mediated control of apoptosis occurs through qualitative rather than quantitative changes in protein synthesis, mediated by a dynamic interplay between cap-dependent and cap-independent processes.

Apoptosis in higher eukaryotes is controlled by a precisely orchestrated interaction among regulatory and effector molecules that function to eliminate unwanted cells during development, an immune response, tissue repair, and oncogenesis. Control points for apoptosis have been identified at different levels of biological regulation, including translational control. The efficiency of utilization of mRNA encoding a number of potent positive and negative regulators of apoptosis is tightly regulated (reviewed in references 4, 5, and 16).

Translational control is exerted predominantly by regulating the quantity, activity and integrity of the cap-dependent translation initiation apparatus (reviewed in references 15, 31, 32, and 33). In mammals, a trimolecular complex designated eukaryotic initiation factor 4F (eIF4F) initiates cap-dependent translation. It consists of three proteins: eIF4E, which binds the 7-methyl guanosine cap at the 5′ mRNA terminus; eIF4A, an mRNA helicase; and an eIF4G family member (eIF4GI or eIF4GII), which serves as a docking protein binding eIF4E and eIF4A. eIF4G also binds the adapter protein eIF3 which targets the intact eIF4F complex to the 40S subunit of the ribosome. Translation can also be initiated in a cap-independent fashion through internal ribosomal entry sites (IRES) (reviewed in references 3, 18and 27). When this occurs, sequences in the 5′-untranslated region of mRNA are capable of directly binding to domains on eIF4G family proteins allowing cap-independent recruitment of the transcript to the 40S ribosomal subunit.

The translational function of eIF4E is negatively regulated by a family of the eIF4E-binding proteins (4E-BPs), which share a motif with eIF4G allowing them to bind and sequester eIF4E in a competitive manner 19, 20, 26; reviewed in references 32 and 33). In this regard, the affinity of the prototype family member 4E-BP1 (also known as PHAS-I) for eIF4E is regulated by the phosphorylation state of six serine/threonine (S/T) residues on the 4E-BP1 molecule (T37, T46, S65, T70, S83, and S112, numbered according to the human 4E-BP1 sequence) (8, 38). In response to exogenous stimuli, such as growth factors and insulin, 4E-BP1 appears to be phosphorylated in a stepwise fashion, with phosphorylation of the two amino-terminal threonines (T37 and T46) functioning together to promote subsequent phosphorylation of the carboxy-terminal sites (9, 10, 24, 25). In addition, phosphorylation at T70 facilitates phosphorylation at S65 (25). Growth factors and hormones signal phosphorylation of 4E-BPs through the Akt/protein kinase B kinase cascade that includes FRAP/mTOR (FKBP12–rapamycin-associated protein or mammalian target of rapamycin) (reviewed in references 12 and 34). FRAP/mTOR mediates phosphorylation of 4E-BP1 at residues T37 and T46 and also impacts phosphorylation of S65 and T70. In

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general, accumulation of phosphate residues on 4E-BP1 decreases its affinity for eIF4E, which becomes free to bind eIF4G and initiate translation (9, 10, 24, 25).

Key components of the translation initiation apparatus have been directly implicated as targets and regulators of apoptosis (reviewed in references 2, 5, and 17). For some time it has been recognized that inhibition of protein synthesis is an early biochemical event in the process of apoptosis (7, 23, 39). At the onset of apoptosis, eIF4G is cleaved by activated caspases and cap-dependent translation abruptly decreases (5, 21). Although it is established that translation of mRNA in a cap-dependent manner is suppressed during apoptosis, this is not the case for translation of transcripts bearing an IRES, which may actually be increased (13, 17). Several studies have identified truncated forms of eIF4G family members that can support protein synthesis in apoptotic cells through an IRES. One eIF4GI cleavage product, an M-FAG (for middle fragment of apoptotic cleavage of eIF4G fragment), contains binding sites for eIF4E and eIF4A and supports some cap-dependent initiation as well as initiation utilizing an IRES (5). It shares 32% homology with a member of the eIF4G protein family, p97/DAP5/NAT1, which lacks the N-terminal eIF4E-binding site and is therefore unable to initiate cap-dependent translation. Like the M-FAG fragment, p97/DAP5/NAT1 can sustain translation of mRNAs containing IRES, including its own mRNA (reviewed in references 9, 11, and 17). Of note, the transcript for p97/DAP5/NAT1 has an IRES, setting up a positive feedback loop as apoptosis eventuates to ensure continued translation of certain proapoptotic IRES-bearing transcripts (13). In fact, p97/DAP5/NAT1 is a potent agonist of apoptosis, and the transcripts for other proapoptotic effectors such as c-Myc and Apaf-1 contain an IRES (17). These data support the notion that among the mRNAs encoding apoptotic regulatory proteins, those suppressing apoptosis are cap dependent, whereas those translated through IRES support the execution of apoptosis (13, 28). In accord with this formulation, ectopic expression of eIF4E rescues cells from apoptosis induced by growth factor restriction or cytostatic drugs (29, 35), while ectopic expression of wild-type 4E-BP1 activates apoptosis in Ras-transformed fibroblasts, leaving nontransformed cells viable (28).

While this body of evidence highlights the importance of translational control in apoptosis, the difference in viability between transformed and nontransformed cells when 4E-BP1 is ectopically expressed remains unexplained. Even a 20-fold increase in 4E-BP1 expression in nontransformed fibroblasts does not activate apoptosis (28). This finding raises the question of whether nontransformed cells are more able than their transformed counterparts to maintain the critical balance of antiapoptotic and proapoptotic proteins needed for viability in the face of increased levels of 4E-BP1 or whether the downstream repertoire of apoptotic regulatory events is fundamentally different after malignant transformation. Therefore, our objective in the present study was to determine whether intensifying repression of cap-dependent initiation by dephosphorylation of 4E-BP1 triggered apoptosis in nontransformed fibroblasts. We employed two complementary approaches: (i) pharmacological blockade of ectopic and endogenous 4E-BP1 phosphorylation with rapamycin and (ii) ectopic expression of 4E-BP1 mutants lacking specific phosphorylation sites. Here, we show that inhibition of 4E-BP1 phosphorylation by rapamycin triggers apoptosis in nontransformed fibroblasts ectopically expressing wild-type 4E-BP1. We also demonstrate that transient or stable expression of phosphorylation site mutants in nontransformed fibroblasts potently activates apoptosis in a phosphorylation-site specific manner but not in strict accord with the mutant forms' ability to repress cap-dependent translation.

MATERIALS AND METHODS

4E-BP1 expression vectors. Single and double 4E-BP1 phosphorylation mutants were generated by using PCR site-directed mutagenesis of human 4E-BP1 sequences cloned into the cytomegalovirus-based pACTAG vector, as described (9, 28). For stable transfection, the original (wild-type) and mutant 4E-BP1 sequences were inserted into the mammalian expression vector pSR *puro* between *Eco*RI and *Bam*HI sites (pSR *puro*/4E-BP1 vector). For transient transfection and clonogenic assays, the wild-type and mutant 4E-BP1 sequences were subcloned into the pACTAG-2 construct containing an amino-terminal three-hemagglutinin (three-HA) tag and a neomycin resistance gene cassette. The resulting vectors (pACTAG *neo*/HA-4E-BP1) were sequenced in their entirety, and the coding regions were found to be free of undesired mutations. To evaluate rates of cap-dependent translation and translation mediated by an IRES, we used a bicistronic reporter plasmid, pcDNA3-rLuc-polIRES-fLuc (28), modified for expression in eukaryotic cells by insertion of the pCMV promoter.

Cell culture, transfection, and clonogenic assay. Cloned rat embryo fibroblasts (CREF) were described previously (28). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS). For both stable and transient transfection, 3.5×10^5 CREF were plated in 35-mm wells of six-well clusters. After 24 h, cells were transfected with 4E-BP1 expression plasmids or with an empty vector using Lipofectamine Plus reagent (Gibco) according to the manufacturer's instructions. To generate clonal cell lines constitutively expressing the wild type or serine-to-alanine (A) mutants of 4E-BP1 at amino acid 65, CREF were transfected with the pSR *puro*/4E-BP1 vector and after 2 days were selected for resistance to 1-μg/ml puromycin (Sigma) for a period of 2 weeks. Individual puromycin-resistant clonal lines were screened for expression of 4E-BP1. For transient gene transfer, cells were transfected with the empty pACTAG *neo*/HA vector or with the pACTAG *neo*/HA-4E-BP1 expression plasmids encoding either wild-type or mutant 4E-BP1. Floating and adherent cells were collected after 48 h and subjected to immunoblotting or flow cytometric assays to detect expression of HA and 4E-BP1 and to quantify apoptotic frequency. Transfection efficiency was quantified by flow cytometric analysis of the percentage of HA-positive cells after incubation of transfected cells with primary HA antibody, followed by staining with fluorescein-conjugated secondary antibody, as described (28). Values for all constructs clustered around 23% (range, 20.3 to 24.9%).

Apoptosis assay. To quantify apoptotic frequency, cells were washed in ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol, followed by incubation with propidium iodide stain mixture (50 μg of propidium iodide/ml, 0.1% Triton X-100, 37 μg of EDTA/ml, 2.5 U of RNase/ml in PBS) for 60 min at room temperature. The percentage of cells with hypodiploid DNA content was determined with a FACScan flow cytometer (Becton Dickinson) with the CellQuest program.

Immunoblot analysis. Floating and adherent cells were collected, washed with cold PBS, and lysed by three successive freeze-thaw cycles in 50 mM Tris, (pH 7.4), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg of pepstatin A/ml, 10 mM sodium pyrophosphate, 50 mM β-glycerolphosphate, and 0.1 mM Na_3VO_4. Cellular proteins (50 μg) were resolved by either an 8 to 15% linear gradient or sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Blots were blocked in TBST with 5% dry milk and 0.05% Tween-20. To detect 4E-BP1, eIF4G1 and eIF4E blots were incubated with antibody, washed, incubated with the appropriate horseradish peroxidase-coupled secondary antibody, and detected by enhanced chemiluminescence as described (28).

Cap affinity binding assay. m^7GTP-Sepharose chromatography was performed as described previously (28) by applying 250 μg of cell lysate protein in a volume of 250 μl to 20 μl of packed m^7GTP-Sepharose beads. Captured proteins were eluted with buffer containing 70 μM m^7GTP and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to transfer and immunoblot analysis.

Quantification of cap-dependent and IRES-mediated translation. Cells were cotransfected with 1 μg of pcDNA3-rLuc-polio-fLuc and either wild-type or mutant pACTAG/HA-4E-BP1 (1 μg) or with 1 μg of pACTAG/HA vector. Cells were rinsed with PBS 24 h after transfection and incubated with the passive lysis buffer (Promega) for 15 min. Cell debris was pelleted by centrifugation, and triplicate supernatant samples were assayed for *Renilla* and firefly luciferase activities in a Lumat LB 9507 luminometer (BG&G, Berthold, Germany) using the Promega dual luciferase reporter system.

Statistics. Statistical analyses were performed using one-way analysis of variance with Dunnett's multiple comparison test (S-PLUS Guide to Statistical and Mathematical Analysis, version 4.0; Insightful, Seattle, Wash.). A P value of <0.05 was considered significant.

RESULTS

Rapamycin and ectopic 4E-BP1 synergistically impair assembly of the cap-dependent initiation complex and activate apoptosis. When wild-type 4E-BP1 is ectopically expressed in nontransformed fibroblasts (CREF), it is unable to activate apoptosis (28). The phosphorylation status of 4E-BP1 modulates its ability to sequester eIF4E and repress cap-dependent translation, with hypophosphorylated forms associating most avidly. To determine if decreasing the phosphorylation status of ectopically expressed 4E-BP1 increased its proapoptotic potency, we developed clonal cell lines of CREF ectopically expressing wild-type 4E-BP1 at different levels and manipulated 4E-BP1 phosphorylation status pharmacologically. Phosphorylation of 4E-BP1 occurs in a FRAP/mTOR kinase-dependent manner, a reaction inhibited in vivo by rapamycin (1, 10, 12, 34). We selected clonal fibroblast lines expressing 4E-BP1 at high or intermediate levels and compared the viability of each to untransfected cells in the presence or absence of rapamycin (Fig. 1). To minimize the effect of extracellular signaling, this set of experiments was conducted in medium containing 0.1% FCS, a condition in which endogenous 4E-BP1 expression is barely detectable.

As expected, addition of rapamycin decreased expression of hyperphosphorylated 4E-BP1 γ and increased the expression of hypophosphorylated 4E-BP1 α (Fig. 1A). The integrity of the eIF4F cap-dependent translation initiation complex was examined using cap analogue capture of eIF4E and its binding partners in cell lysates, followed by immunoblot analysis. This analysis revealed no change in eIF4E partnering after rapamycin treatment of nontransfected cells; eIF4GI remained the predominant binding partner of eIF4E (Fig. 1B). In the clonal line expressing intermediate levels of 4E-BP1, there was no detectable decrease in the binding of eIF4G1 to eIF4E. Rapamycin inhibited phosphorylation of 4E-BP1 and displaced eIF4GI from eIF4E, indicating disassembly ofeIF4F. In the high-level expressors, where 4E-BP1 predominated as the binding partner of eIF4E, rapamycin caused a discernible bias toward hypophosphorylated 4E-BP1 bound to the cap-captured eIF4E. Of note, in association with these biochemical changes, rapamycin significantly increased the frequency of apoptosis in cells ectopically overexpressing 4E-BP1 (Fig. 1C and D). In accord with our previously published findings, ectopic expression of 4E-BP1 in the absence of rapamycin did not trigger apoptosis. Thus, pharmacological inhibition of 4E-BP1 phosphorylation activates apoptosis in nontransformed fibroblasts ectopically expressing wild-type 4E-BP1.

Mutating Ser-65 to Ala increases the proapoptotic activity of ectopically expressed 4E-BP1. To examine the relationship between the expression level of hypophosphorylated 4E-BP1 and susceptibility to apoptosis, we developed a series of clonal cell lines stably expressing a serine-to-alanine mutation of 4E-BP1 at residue 65 (designated 4E-BP1A65). We selected this residue, since phosphorylation of Ser-65 is rapamycin sensitive and has the least impact on phosphorylation of other S/T residues in the 4E-BP1 molecule (8, 9, 10, 25). Four clonal cell lines of A65 were chosen that represented a 60-fold range of expression, and displayed mostly hypophosphorylated α and β forms (Fig. 2A). Apoptosis was quantified under standard culture conditions after serum withdrawal and after exposure to either genotoxic (e.g., camptothecin) or nongenotoxic (e.g., lovastatin) cytostatic agents. While the magnitude of the apoptotic response differed among the stimuli examined (with serum withdrawal exerting the most potent proapoptotic effect, followed by lovastatin and camptothecin), there was a reproducible rank order of susceptibility to apoptosis that exactly paralleled the level of ectopic 4E-BP1A65 expression (Fig. 2B). Apoptotic frequency ranged from 2 to 18% for the mock-transfected line, peaking at 17 to 44% for the highest expressor examined. Since even highly overexpressed wild-type 4E-BP1 does not activate apoptosis in this cell system, these data suggest that mutating Ser-65 to alanine is sufficient to convert 4E-BP1 from a nonapoptotic to a proapoptotic form for CREF. They also establish a direct relationship between the level of mutant 4E-BP1 expressed and fibroblast susceptibility to apoptosis.

To examine the impact of increasing cellular levels of 4E-BP1A65 in the absence of clonal selection, fibroblasts were transiently transfected with expression vectors that included HA epitope-tagged wild-type or 4E-BP1A65 sequences. Both HA expression and DNA content were quantified by using a two-parameter flow cytometric analysis. Analysis of nontransfected fibroblasts revealed that the basal frequency of apoptosis (hypodiploid DNA content) was approximately 3% (2.4% in the example shown in Fig. 2C) and permitted calibration of the background HA signal in the apoptotic cell population (mean signal intensity = 7.3, expressed in arbitrary units). Expression of HA vector increased the frequency of apoptosis to 6.9% and, as expected, increased the HA signal intensity in all cells analyzed, independent of DNA content. Introduction of wild-type 4E-BP1 into fibroblasts yielded an apoptotic frequency similar to that seen with empty vector. There was, however, a significant skew of hypodiploid cells toward HA positivity (mean signal intensity = 36.8), indicating increased DNA degradation in cells harboring exogenous 4E-BP1. Transfection with the A65 mutant doubled the frequency of apoptotic cells, with the vast majority being HA bright (mean signal intensity = 35.9). Of note, the HA-bearing constructs resulted in a very narrow range of HA positivity (20.7 to 24.4%), suggesting that differences in transfection efficiency did not account for the results observed. These data confirm the proapoptotic activity of 4E-BP1A65 in nontransformed fibroblasts.

Regulation of cell viability by 4E-BP1 is phosphorylation site specific. To comprehensively examine the impact of 4E-BP1 phosphorylation state on fibroblast growth, we set out to quantify the colony-forming efficiency of fibroblasts transfected

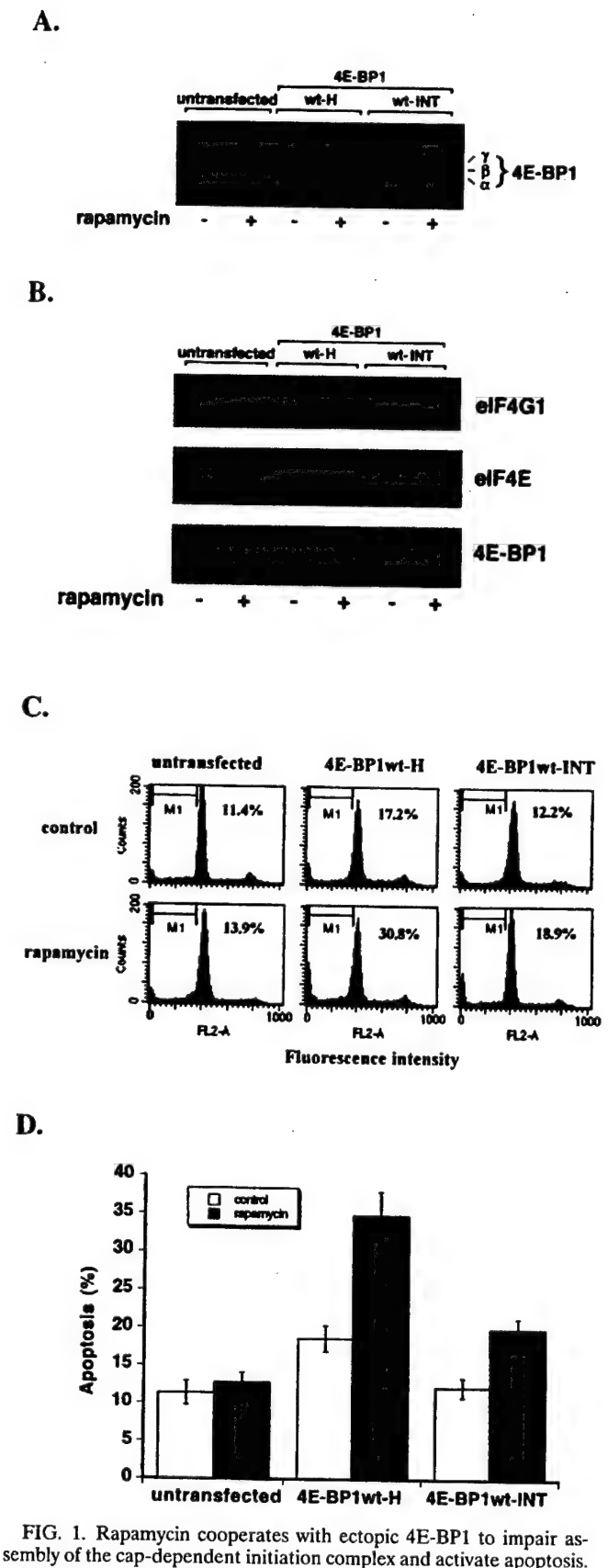

FIG. 1. Rapamycin cooperates with ectopic 4E-BP1 to impair assembly of the cap-dependent initiation complex and activate apoptosis. CREF were stably transfected with untagged wild-type (wt) 4E-BP1. Eight neomycin-resistant clones were developed and analyzed by immunoblotting, and two clonal cell lines were expanded, one expressing high (wt-H) and one expressing intermediate (wt-INT) levels of ectopic 4E-BP1. Nontransfected CREF and cells ectopically expressing 4E-BP1 were preincubated with DMEM plus 0.1% FCS for 18 h, and cultures were continued in the presence or absence of 75 nM rapamycin for 6 (A and B) or for 24 (C and D) h. (A) Immunoblot analyses of steady-state 4E-BP1 expression. (B) Immunoblot analysis of 4E-BP1 and eIF4G1 associated with eIF4E bound to m^7GTP-Sepharose. (C) Flow cytometric data from a representative experiment. The percent hypodiploid DNA is indicated in the upper right corner of each plot. (D) Apoptotic frequency (hypodiploid DNA content determined by flow cytometry). The mean ± standard deviation (SD) of three independent experiments is shown.

with a neomycin resistance cassette bearing sequences for wild-type 4E-BP1 or S/T-to-Ala mutants at the phosphorylation sites indicated (Fig. 3A). We employed three mutant forms of 4E-BP1: one double mutant (A37/A46) to eliminate phosphorylation N terminal to the eIF4E binding site, and two single mutants, A65 and A70, at phosphorylation sites C terminal to the eIF4E binding site. To detect any potential experimental bias resulting from systematic differences in ectopic wild-type or mutant 4E-BP1 expression due to self repression (i.e., negative feedback from the ectopic protein itself leading to decreased translation of ectopic and/or endogenous 4E-BP1 despite identical gene transfer efficiency), cells were transiently transfected in parallel with a pACTAG *neo* vector encoding wild-type or mutant HA-tagged 4E-BP1 using identical procedures in four independent experiments. Immunoblot analysis of 4E-BP1 expression showed equivalency for endogenous 4E-BP1 and all forms of HA-tagged 4E-BP1, except for cells transfected with A37/A46. For this double mutant, endogenous and exogenous 4E-BP1 was reproducibly expressed at a lower level (Fig. 3B). Replication of this experiment employing a retroviral gene transfer procedure led to similar results (not shown). To detect any experimental bias due to systematic differences in gene transfer efficiency, flow cytometric analysis was carried out. The proportion of cells expressing each mutant form (i.e., HA bright) was similar, ranging from 20.3 to 24.9% (not shown). These data indicated that self-repression, not differences in the proportion of transfected cells, most likely accounted for decreased expression of the A37/A46 mutant.

After transfection of CREF with empty vector, the wild type, and each mutant form, cultures were continued for 2 weeks and colony-forming efficiency was scored as the number of G418-resistant colonies produced by fibroblasts transfected by each HA-tagged 4E-BP1 vector, normalized to colony counts after transfection with empty HA vector. Ectopic expression of wild-type 4E-BP1 did not significantly alter CREF colony formation (Fig. 4A and B), a result in accord with its lack of impact on apoptosis in nontransformed fibroblasts (28). In sharp contrast, colony formation by fibroblasts transfected with S/T phosphorylation mutants was significantly reduced, ranging from 20% fewer colonies in response to transfer of the A37/A46 mutant to an 80% reduction in colony number after transfer of the A70 mutant. Values for A65 were intermediate.

The reduction of colony formation observed could have resulted from inhibition of cell cycle transit, activation of cell death, or both. We explored these possibilities by subjecting

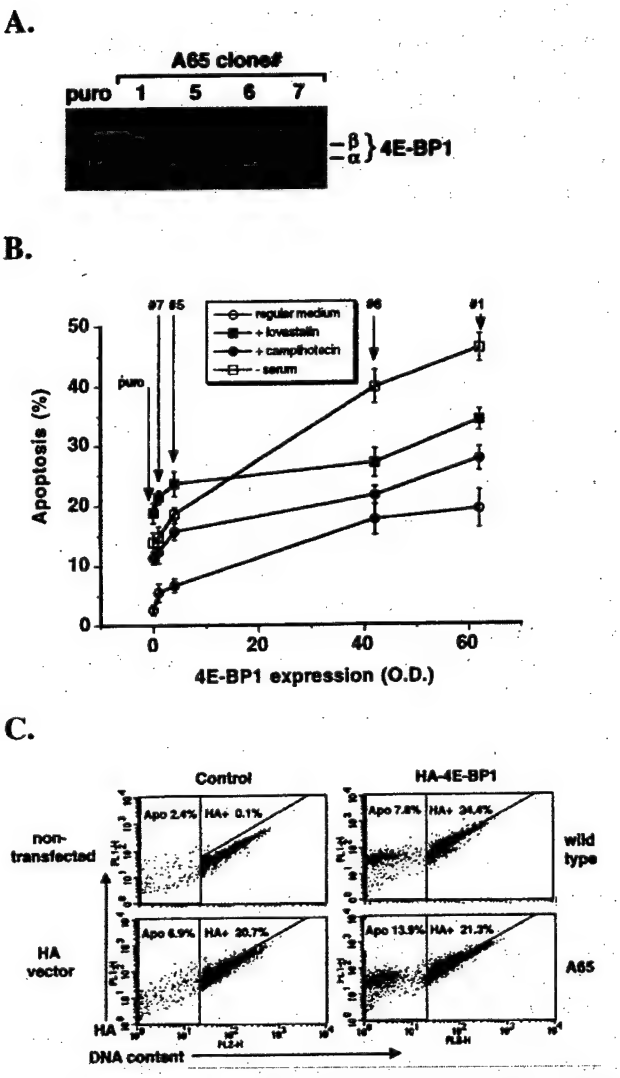

FIG. 2. Activation of apoptosis by 4E-BP1A65 depends on its level of expression. CREF were stably transfected with a construct encoding an untagged A65 mutant of 4E-BP1. Eleven neomycin-resistant clones were isolated, and expression of 4E-BP1 was assessed by immunoblotting. Four clonal cell lines expressing a range of ectopic 4E-BP1A65 were propagated. (A) Immunoblot analysis of 4E-BP1 expression in A65 clones 1, 5, 6, and 7 compared to a clonal line bearing an empty puromycin vector (puro). (B) Apoptosis assay. Cells were cultured for 48 h in serum-free medium or incubated for 24 h in growth medium (DMEM plus 10% FCS) with or without 5 μM lovastatin or 300 nM camptothecin. Cells were fixed and stained with propidium iodide, and the percentage of cells with hypodiploid DNA content was quantified by flow cytometry. The results shown represent the mean ± SD of three independent experiments. (C) Two-parameter flow cytometric analysis of HA–4E-BP1 expression and apoptosis. CREF were transiently transfected with pACTAG encoding the indicated form of HA–4E-BP1 or with empty pACTAG. Cells were fixed with 1% formaldehyde, postfixed with 70% ethanol, exposed to fluorescein-conjugated anti-HA antibody (5 μg/ml), and stained with propidium iodide. HA expression (green fluorescence) (vertical axis) and DNA content (red fluorescence) (horizontal axis) were quantified in controls (nontransfected, upper left; empty HA vector, lower left) and after transfection with either HA–4E-BP1wt (upper right) or the A65 mutant (lower right). Gating parameters are as follows: the diagonal line in each panel defines the boundary between HA-positive and -negative cells, and the vertical line in each panel defines the hypodiploid DNA boundary. The result of a representative experiment is shown (three independent experiments yielded similar results).

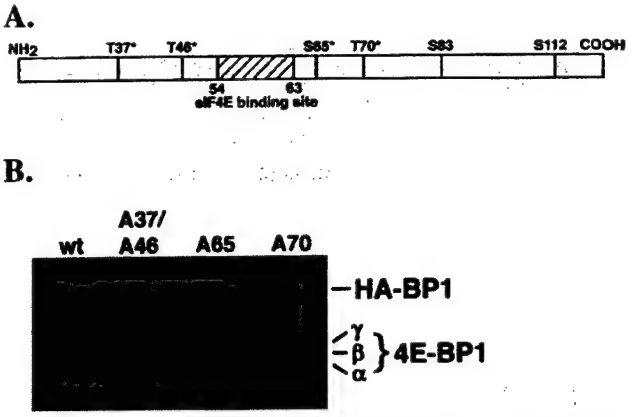

FIG. 3. Steady-state levels of endogenous and ectopic 4E-BP1 after transfection with wild-type or mutant forms of 4E-BP1. (A) Shown are the positions of the four serine/threonine phosphorylation sites of 4E-BP1 mutated to alanine relative to the eIF4E binding site (designated with asterisks), and serines 83 and 112, which were left nonmutated in our studies. (B) Immunoblot assessment of 4E-BP1 expression. CREF were transfected with 1 μg of empty pACTAG or with 1 μg of pACTAG encoding either wild-type 4E-BP1 (wt), or one of indicated S/T-to-alanine 4E-BP1 mutants (all HA epitope tagged). Cells were analyzed for steady-state expression of ectopic (HA-BP1) and endogenous (phosphorylation forms α, β, and γ are shown) 4E-BP1. Presented is a blot representative of four independent transfection experiments.

CREF to flow cytometric analysis after transient transfection with constructs encoding wild-type or mutant HA-BP1. The proportion of cycling cells (S phase plus G_2/M) was not significantly altered by any of the 4E-BP1 constructs (Fig. 4C), whereas the proportion of cells with hypodiploid DNA content (apoptotic cells) was increased from 1.7- to 2.5-fold by the phosphorylation site mutants but not by the wild-type 4E-BP1. Among wild-type and mutant forms, the rank order of potency in activating DNA degradation generally paralleled the potency in suppressing colony formation with A70 > A65 > A37/A46 > wild type. These data suggest that the 4E-BP1 phosphorylation site mutants suppressed CREF colony formation by activating apoptosis and not by blocking cell cycle transit. However, these results leave open the question of whether the relatively low potency of the A37/A46 mutant was a true reflection of its decreased intrinsic ability to modulate cell fate or simply resulted from insufficient expression.

Relationships among cap-dependent translation, translation mediated by an IRES, and fibroblast viability. We next investigated whether the rank order of proapoptotic potency of each 4E-BP1 phosphorylation site mutant matched its potency in repressing cap-dependent protein synthesis. 4E-BP1-induced alterations of cap-dependent and IRES-mediated translation were detected by cotransfecting fibroblasts with wild-type HA–4E-BP1 or mutant constructs, along with a bicistronic reporter vector encoding *Renilla* and firefly luciferases. In this system, translation of *Renilla* luciferase was cap dependent, whereas translation of firefly luciferase was mediated by an IRES (30).

Expression of either wild-type or mutant 4E-BP1 suppressed cap-dependent translation with a rank order of inhibitory potency conforming to previously published results (25): A37/A46 > A70 > A65 > wild type (Fig. 5A). In this regard, the

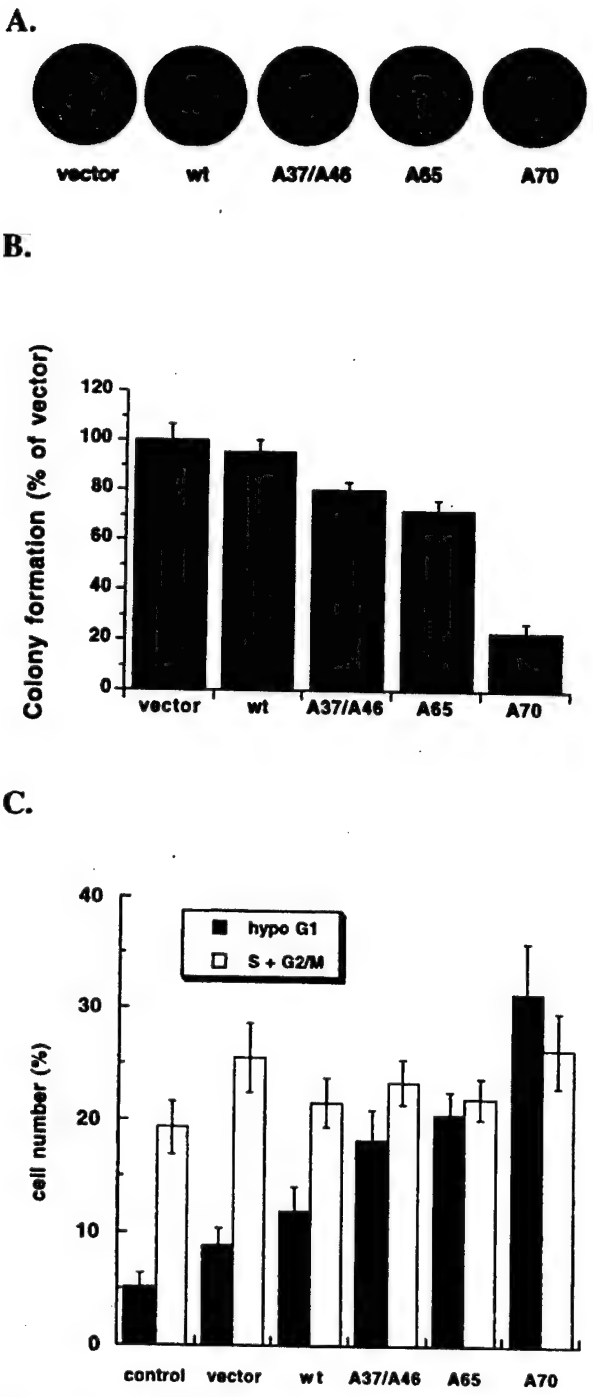

FIG. 4. Mutating 4E-BP1 phosphorylation sites reduce fibroblast colony formation and increase apoptosis. CREF were transfected either with vector, the 4E-BP1 wild type (wt), or mutant forms of 4E-BP1. (A and B) Transfected cells were seeded at low density and cultured for 2 weeks. G418-resistant colonies were scored after fixation with 10% formaldehyde and Coomassie staining. Shown is a photograph of a representative set of dishes (A) and colony counts (B), expressed as the ratio of the number of colonies formed by cells transfected with the indicated 4E-BP1 to the number of colonies formed by cells transfected with empty pACTAG vector (results represent the mean ± SD of three independent experiments). (C) DNA content after ectopic expression of wild-type or mutant 4E-BP1. After the transfection procedure, cells were fixed with 70% ice-cold ethanol and stained with propidium iodide, and the frequency distribution of red fluorescence was defined. Shown is the proportion of nontransfected and transfected cells exhibiting hypodiploid DNA content (hypo G_1), taken as an index of apoptosis, or hyperdiploid DNA content (S + G_2/M), taken as a measure of proliferation.

viability index [(1 − % hypodiploid 4E-BP1 transfected cells)/(1 − % hypodiploid vector transfected)] followed cap-dependent translational repression for the A70, A65, and wild-type forms of 4E-BP1 (Fig. 5B), which left IRES-mediated translation unaltered. In contrast, while A37/A46 clearly exerted the most potent repression of cap-dependent translation despite a lower level of ectopic protein expression (70% inhibition compared to vector control), it was the least potent of the tested mutants as an activator of apoptosis and significantly stimulated translation via the IRES. Thus, our data indicate that the relationship between 4E-BP1-induced inhibition of cap-dependent translation and reduction of cell viability is not monotonic.

To distinguish an effect resulting from mutating residues 37 and 46 from one due to the alanine substitutions per se, we studied colony-forming efficiency, apoptosis, and translational repression of fibroblasts ectopically expressing 4E-BP1 E37/E46, in which T37 and T46 were mutated to glutamine. We found that ectopic expression of this mutant form gave results very similar to its alanine-substituted counterpart, although it was slightly less active in suppressing colony formation, activating apoptosis, suppressing cap-dependent translation, and activating translation via an IRES (not shown). Therefore, our data indicate that the proapoptotic effects observed were due to elimination of the N-terminal phosphorylation sites and their resultant impact on translation initiation.

DISCUSSION

Cell fate is subject to translational control (2, 4, 6, 16, 17), a principle that is underscored by the wide variety of human tumors and cancer cell lines in which the cap-dependent initiation apparatus is activated (reviewed in references 6 and 16). In prior studies, overexpressed eIF4E was found to prevent the death of both nontransformed and transformed fibroblasts, whereas wild-type 4E-BP1 promoted apoptosis only in transformed cells, sparing nontransformed fibroblasts (28, 29). This raised the question of whether malignant transformation fundamentally alters translational control of apoptosis or whether it serves to increase the cellular requirement for cap-dependent translation. Here, we show that nontransformed fibroblasts undergo apoptosis when repression of cap-dependent translation is intensified. Transfer of wild-type 4E-BP1 into nontransformed fibroblasts together with pharmacological blockade of 4E-BP1 phosphorylation using the FRAP/mTOR kinase inhibitor rapamycin activated apoptosis. Mutation of 4E-BP1 at residues T37, T46, S65, or T70 eliminates phosphorylation sites, resulting in mutant proteins that are able to evade physiological mechanisms of deactivation. Transfer of these mutant forms into nontransformed fibroblasts markedly decreased their ability to form colonies and increased apoptosis in a manner dependent on the presence of specific phosphorylation sites. In general, for the wild type and each mutant 4E-BP1 protein studied, we found that proapoptotic potency paralleled its ability to repress cap-dependent translation. Sur-

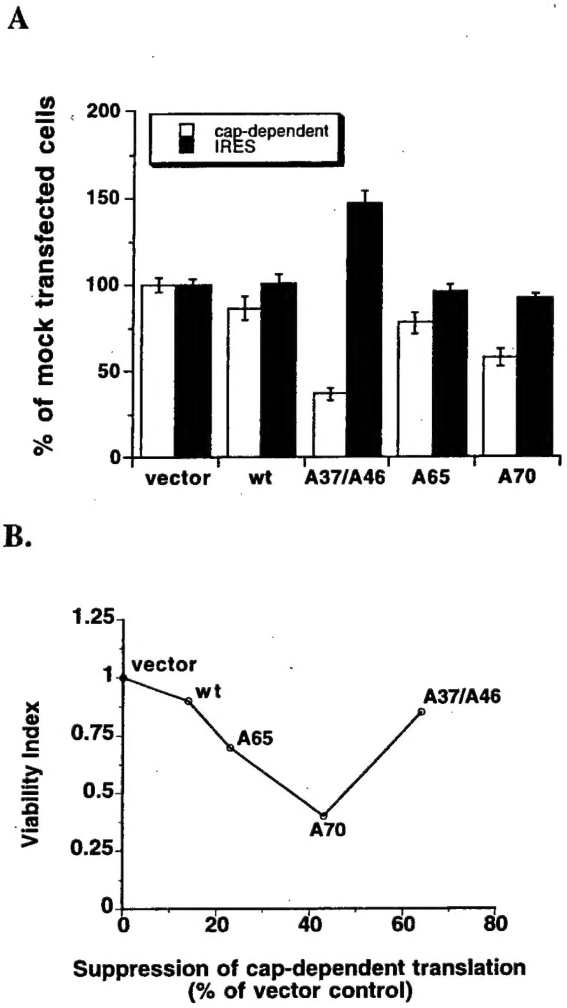

FIG. 5. Relationships among cap-dependent translation, translation mediated by an IRES, and fibroblast viability. Cells were cotransfected with pACTAG encoding wild-type or mutant HA–4E-BP1 and the pcDNA3-rLuc-polio-fLuc plasmid. (A) Cap-dependent and IRES-directed luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). The mean ± SD from three independent experiments is shown. (B) Viability as a function of cap-dependent translation. The viability index was calculated from flow cytometric data as follows: (1 − % hypodiploid 4E-BP1 transfected cells)/(1 − % hypodiploid vector transfected cells). Mean values from three independent experiments are presented.

prisingly, our data did not reveal a strict monotonic relationship between cap-dependent translation and apoptosis. Instead, we found that as repression of cap-dependent translation intensified, apoptosis increased to a maximum value (observed with the A70 mutant) and that further repression (achieved with A37/A46) resulted in less apoptosis—a state associated with activation of translation through an IRES. Together with our previous observations (28), these findings reveal that 4E-BP1 is proapoptotic in both normal and transformed fibroblasts and suggest that oncogenic transformation may confer cells with a markedly reduced capacity to block apoptosis when cap-dependent translation is suppressed by 4E-BP1. Our results also suggest that 4E-BP1-mediated control of apoptosis occurs through qualitative rather than quantitative changes in protein synthesis, mediated by a dynamic interplay between cap-dependent and cap-independent processes.

Excluding the limiting case where an insufficient quantity of structural proteins or critical enzymes is synthesized to sustain life, the role of translational control in the regulation of apoptosis is just coming into focus. In some cell types including mammalian neurons (7, 22) and fibroblasts (28), pharmacological inhibition of peptide chain elongation suppresses programmed cell death. In contrast, specific inhibition of cap-dependent translation initiation by ectopic expression of 4E-BP1 stimulates apoptosis in Ras-transformed fibroblasts in a manner dependent upon its ability to sequester eIF4E (28). Consistent with this, ectopic expression of eIF4E rescues fibroblasts from apoptotic death (29). Here, we add to the data connecting the cap-dependent initiation apparatus to the regulation of apoptosis. Ectopic expression of 4E-BP1 in nontransformed fibroblasts (CREF) had no discernible impact on the assembly of eIF4F or on cell viability. However, inhibition of phosphorylation with rapamycin led to disassembly of eIF4F and activation of apoptosis. In accord with this result, introduction of mutant forms of 4E-BP1 lacking specific phosphorylation sites into CREF also activated apoptosis. These findings suggest that the FRAP/mTOR kinase cascade, as well as other pathways leading to 4E-BP1 phosphorylation, is an important component of the antiapoptotic signaling system activated by extracellular survival factors. In addition, these data demonstrate a close connection between cellular functions controlled by 4E-BP1 and the regulation of apoptosis, thus supporting the concept that activation of cap-dependent translation leads to the synthesis of regulatory proteins that antagonize apoptosis.

A detailed examination of the relationship between repression of cap-dependent initiation and apoptosis proved informative. The mutant forms of 4E-BP1 studied suppressed cap-dependent initiation in exactly the rank order of potency expected based on prior publications (A70 > A65 > A37/A46 > wild type) (9, 10, 24, 25). In general, activation of apoptosis followed this same pattern, with A70 > A65 > wild type. Only A37/A46 deviated from expectation, a result with several important implications. First, despite being expressed at lower levels than the other 4E-BP1 forms, A37/A46 suppressed translation most robustly. This dissociates the level of ectopic protein expression from the intensity of translational repression and strongly suggests that our findings do not result from toxicity of the mutant proteins themselves. Second, introduction of the double mutant (A37/A46) allowed us to reduce cap-dependent translation initiation by 70%, which by itself was not sufficient to achieve maximum levels of 4E-BP1-induced apoptosis. This makes it unlikely that the apoptosis observed was triggered by metabolic poisoning due to inadequate protein synthesis—pointing strongly instead to a change in the balance of factors regulating apoptosis, not those required to sustain other critical physiological functions. Third, in cells expressing the A37/A46 mutant, translation via IRES was activated concomitantly with a decline in apoptosis. Somewhat surprisingly, rather than leading to potentiation of apoptosis as might be expected from the simplest model of translational control (that cap-dependent translation rescues, and

translation via IRES promotes, apoptosis), apoptosis was actually attenuated.

The data in this report together with the published literature lead us to speculate that at some threshold level, the proapoptotic effect of hypophosphorylated 4E-BP1 may be mitigated by concomitant activation of IRES-mediated translation of rescue proteins. In this connection, experimental precedent does exist for translation of known rescue moieties via IRES, including insulin-like growth factor I receptor, insulin-like growth factor II, and X-linked inhibitor of apoptosis protein (17). Along these lines, recent findings indicate that the potent mediator of IRES-dependent translation, p97/DAP5/NAT1, is a bifunctional regulator of neuroblastoma cell fate—essential for both viability and apoptosis (37). Alternatively, as repression of cap-dependent translation is intensified, apoptosis may be attenuated by a reduction in cap-dependent death proteins independent of any effects exerted by proteins translated using IRES.

Our prior studies (28, 29, 35) together with the data reported here establish the functional import of regulating cap-dependent translation initiation in the control of programmed cell death. In addition, as the process of apoptosis unfolds, the translational machinery itself is attacked, setting up potential positive or negative feedback loops. For example, eIF4GI and eIF4GII are cleaved by factors triggering, controlling, or executing apoptosis (5, 21), resulting in shutdown of cap-dependent protein synthesis. In addition to the eIF4G family proteins, 4E-BP1 is also cleaved during apoptosis (2, 36). In accord with the general concept that cap-dependent translation machinery is suppressed during apoptosis (2, 4), one of the 4E-BP1 daughter fragments retains the ability to sequester eIF4E (36). In this regard, recent work demonstrates that sequestration of eIF4E by overexpressed 4E-BP1 (28) or by synthetic oligopeptides that associate with its eIF4G–4E-BP1 binding motif (14) is proapoptotic. These findings indicate that the cap-dependent initiation apparatus is both a regulator and target of the apoptotic machinery and suggest that degradation of specific components of the initiation complex is an integral part of apoptotic death.

Based on current experimental evidence, it appears possible that cell fate is in part determined by a dynamic interplay between cap-dependent translation and translation via IRES. The level and activity of the 4E-BP family of proteins are major determinants of the rate of translation initiation utilizing eIF4F, the cap binding apparatus. Thus, this formulation assigns a central role to the 4E-BP family of proteins and the biochemical pathways governing their phosphorylation status in the control of apoptosis. It also highlights the need for studies in which cap-dependent and IRES-mediated translation is independently manipulated—to elucidate the rules governing which mechanism is utilized in the translation of critical mRNA species encoding proteins that regulate cell viability.

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